REMARKS

Upon entry of the present amendment, claims 1-13 are pending in the application. No claim amendments have been presented herein. Accordingly, no new matter has been added by this filing.

I. Rejections under 35 U.S.C. § 103

Claims 1-13 have been rejected as being unpatentable over U.S. Patent No. 6,011,040 by Muller *et al.* ("Muller") in view of International Publication WO 98/19690 by Smith *et al.* ("Smith").

The Examiner has indicated that Muller describes a composition comprising reduced folate in a dose range between 0.001 mg and 1000 mg and also comprising vitamin B12 in a dosage range between 0.001 mg and 0.5 mg, while Smith describes compositions that are useful in treating Alzheimer's disease or occlusive vascular diseases and contain folic acid and vitamin B12 in a ratio from about 0.1:1 to about 50:1. According to the Examiner, "one having ordinary skill in the art would have been motivated to employ a natural isomer of reduced folate ... to arrive at the claimed composition such that the pharmacological activity of said composition would be greatly enhanced." (Office Action, page 4).

Applicants traverse. The claimed compositions contain a reduced folate compound and a cobalamin compound such that the reduced folate compound and the cobalamin are present in a ratio of 125:1. Muller, however, fails to describe or suggest a composition that includes reduced folate and cobalamin in a ratio of 125:1. In the only Example that describes a pharmaceutical preparation that contains both a reduced folate compound (5-methyl-(6S)-tetrahydrofolic acid) and a cobalamin compound (vitamin B_{12}), the ratio of reduced folate to cobalamin is 0.4 mg of 5-methyl-(6S)-tetrahydrofolic acid to 0.002 mg of vitamin B_{12}, *i.e.*, a ratio of 200:1 (*See* Muller, Example 10 at col. 5, lines 9-19).

The Smith reference fails to remedy the deficiencies in the teachings of Muller. In particular, the Smith reference describes compositions that contain folic acid or folate, rather than a reduced folate compound. There is no teaching or suggestion in this reference that would motivate the skilled artisan to include reduced folate rather than folic acid (or folate) in the Smith compositions. Moreover, the ratio of folic acid (or folate) to a vitamin B compound in the Smith compositions is less than the 125:1 ratio required by the claimed compositions. In particular, the Smith compositions contain a ratio of folic acid or folate to vitamin B12 that is between 0.1:1

and 50:1 and preferably from about 0.2:1 to about 25:1. (*See e.g.*, Smith at page 10, lines 3-6). Thus, Smith does not disclose or suggest compositions that contain reduced folate and cobalamin, wherein the ratio of reduced folate to cobalamin is as high as 125:1.

Applicants submit that it would not have been obvious for the skilled artisan to replace the folic acid used in the Smith compositions with a reduced folate compound to arrive at the claimed compositions. As acknowledged by the Examiner on page 2 of the Office Action, the factual inquiries to be considered in establishing a *prima facie* case of obviousness, as set forth in *Graham v. John Deere Co.*, include consideration of secondary considerations such as unexpected results. The claimed compositions provide an unexpected result. As described throughout the as-filed specification, the compounds of the claimed invention are "better tolerated" than compounds that contain folic acid. (*See* specification at page 6, lines 21-22). Thus, the claimed compositions can be administered over a longer period of time without adverse symptoms such as gastrointestinal distress, which is often associated with the administration of folic acid. (*See e.g.*, specification at page 5 lines 2-5).

In addition, the claimed compositions avoid other adverse side effects associated with the administration of folic acid. For example, Dr. Jacob Selhub, a named inventor on the instant application, has shown that excessive folic acid intake leads to adverse side effects such as reduced natural killer cell (NK) cytoxicity. (*See e.g.*, Troen *et al.*, J. Nutr., vol. 136: 189-194 (2006), copy enclosed herewith). NK cells are important in fighting viral infections and have also been shown to kill cancer cells.

Thus, the use of reduced folate in the claimed composition provides the unexpected result of producing a composition that is better tolerated than a composition that contains folic acid or folate. Accordingly, Applicants believe that it would not have been obvious to skilled artisan to replace the folic acid used in the Smith compositions with a reduced folate compound.

Moreover, Applicants submit that it would not have been obvious to reduce the ratio of reduced folate to cobalamin in the Muller compositions to produce the claimed compositions. There is no teaching or suggestion in either Muller or Smith, alone or in combination, that would motivate a person of ordinary skill in the art to modify the Muller by using a lower ratio of reduced folate to cobalamin. In addition, there is no disclosure in these references, alone or in combination, that would provide the skilled artisan with a reasonable expectation that lowering the dosage of reduced folate in comparison to cobalamin would successfully produce a biologically active composition.

Accordingly, the claimed compositions are not obvious over the Muller and Smith references, either alone or in combination. As such, this rejection should be withdrawn.

CONCLUSION

Applicants submit that the application is in condition for allowance and such action is respectfully requested. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact any of the undersigned at the telephone number provided below. The Commissioner is hereby authorized to charge any additional fees that may be due, or credit any overpayment of same, to Deposit Account No. 50-0311, Reference No.21629-004.

Respectfully submitted,

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Unmetabolized Folic Acid in Plasma Is Associated with Reduced Natural Killer Cell Cytotoxicity among Postmenopausal Women[1]

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ABSTRACT Folic acid (FA) supplements and food fortification are used to prevent neural tube defects and to lower plasma homocysteine. Through exposure to food fortification and vitamin supplement use, large populations in the United States and elsewhere have an unprecedented high FA intake. We evaluated dietary and supplemental intakes of folate and FA in relation to an index of immune function, natural killer cell (NK) cytotoxicity, among 105 healthy, postmenopausal women. Among women with a diet low in folate (<233 μg/d), those who used FA-containing supplements had significantly greater NK cytotoxicity ($P = 0.01$). However, those who consumed a folate-rich diet and in addition used FA supplements > 400 μg/d had reduced NK cytotoxicity compared with those consuming a low-folate diet and no supplements ($P = 0.02$). Prompted by this observation, we assessed the presence of unmetabolized FA in plasma as a biochemical marker of excess FA. Unmetabolized folic acid was detected in 78% of plasma samples from fasting participants. We found an inverse relation between the presence of unmetabolized FA in plasma and NK cytotoxicity. NK cytotoxicity was ~23% lower among women with detectable folic acid ($P = 0.04$). This inverse relation was stronger among women ≥ 60 y old and more pronounced with increasing unmetabolized FA concentrations (P-trend = 0.002). Because of the increased intake of FA in many countries, our findings highlight the need for further studies on the effect of long-term high FA intake on immune function and health. J. Nutr. 136: 189–194, 2006.

KEY WORDS: • *carcinogenesis* • *folic acid* • *immune function* • *natural killer cells*

Folic acid (FA)[4] is the parent compound of folate coenzymes whose crucial role in one-carbon transfers for the synthesis of thymidylate, purines, and biological methylation reactions renders this vitamin essential for health and well-being throughout life. The importance of adequate folate intake to public health is underlined by evidence that neural tube defects can be prevented by periconceptional intake of supplemental FA, as well as epidemiologic data linking the prevalence of cancer, cardiovascular, and other diseases to poor folate intake and status. In light of such data, the United States government mandated the fortification of flour and cereal grain products with FA (1).

The perceived safety of regular FA intake (2,3), and the clear benefits of food FA fortification for the reduction of the incidence of neural tube defect and homocysteine-lowering (4–8) lend support to calls to increase food FA fortification to even higher levels (9). However, few data exist on the potential effect of long-term high FA intake with respect to potentially harmful health outcomes. Such evaluation is necessary because current levels of food FA are in excess by as much as twice the target set for fortification (10–12). Fortification comes on top of consumption by as much of 35% of the U.S. population of unregulated over-the-counter vitamin pills containing folic acid (13) and the availability of many breakfast cereal products that are also enriched with FA by as much as 400 μg FA/serving.

Concerns about exposure of the population to excessive FA intake have focused on the known risk of masking the hematological symptoms of vitamin B-12 deficiency, which may also lead to neurological disease (14,15). It was mainly for this reason that the Institute of Medicine recommended an Upper Limit for FA intake of 1 mg/d for adults (16). It is more difficult to define the scope of other potentially adverse effects of excessive FA intake (17). For example, there is concern that excess folate may enhance the development and progression of already existing, undiagnosed premalignant and malignant lesions (18). However, the currently available human data are

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[4] Abbreviations used: DHF, dihydrofolate; DHFR, dihydrofolate reductase; E:T, effector-to-target cell ratio; FA, folic acid; NK, natural killer cell; PI, propidium iodide; THF, tetrahydrofolate.

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insufficient to evaluate this possibility. One way of addressing this problem is to examine the relation of folate status to various health outcomes in existing studies.

In light of such concerns, we evaluated dietary and supplemental intakes of folate and FA in relation to an index of immune function, natural killer cell (NK) cytotoxicity. NK cells are important in fighting viral infections and can also kill cancer cells (19). Prompted by preliminary findings that high supplementary FA intakes were associated with reduced NK cytotoxicity among some women, we assessed the presence of unmetabolized FA in plasma as a biochemical marker of excess FA intake (20–22), and determined its relation to NK cytotoxicity.

SUBJECTS AND METHODS

Study population. The study population was described previously (23). Briefly, participants (n = 105) were a subset (who met eligibility criteria for participation in a study of immune function) of a study population of women in the greater Seattle area recruited for an exercise intervention trial during 1998–2000 (24,25). Eligibility criteria were as follows: postmenopausal; age 50–75 y; in good health; nonsmoking; sedentary; no hormone-replacement therapy in the past 6 mo; alcohol consumption <2 drinks/d (~26 g); BMI between 25 and 40 kg/m^2 or BMI 24.0–24.9 kg/m^2 if body fat > 33% by bioelectric impedance; no history of invasive cancer, diabetes, cardiovascular disease, asthma; no current serious allergies; no regular (≥2 times/wk) use of aspirin or other nonsteroidal anti-inflammatory medications; no use of corticosteroids or other medications known to affect immune function. We also excluded study participants with a reported energy intake <600 kcal/d (2.510 MJ) or > 4000 kcal/d (16.736 MJ) (n = 4), because nutrient calculations in this range are not reliable.

This study reports the relation between dietary folate intakes or unmetabolized FA and NK cytotoxicity, all measured before entering any intervention. The study procedures were approved by the Fred Hutchinson Cancer Research Center's Institutional Review Board and all study participants provided written informed consent.

Dietary and supplemental folate intakes. Nutrient intakes from dietary sources were obtained at the time of entry into the study using a 120-item FFQ designed and validated at the Fred Hutchinson Cancer Research Center (26,27). Intake of individual nutrients was calculated using algorithms for nutrient calculation from the University of Minnesota Nutrition Coding Center nutrient database (26,28). The calculation of folate intake was based on revised values of the database reflecting the fortified levels.

Use of nutritional supplements was ascertained during an in-person interview. Study participants were asked to bring all nutritional supplements currently used to the clinic visit. Labels of the supplements were photocopied, abstracted, and data entered. The number of months the supplements were used during the past 12-mo period was recorded, as was the number of pills per week or day. From these data, the current daily FA intake from nutritional supplements was calculated. Only supplements that were used at least 1 time/wk during the past 3 mo were included.

Questionnaires were used to collect information on demographic and other factors that may affect immune function.

Folate and FA assays. Plasma concentrations of 5-methyltetrahydrofolate (THF), unmetabolized FA, and total plasma folate were measured in fasting subjects in 2003 by combined affinity HPLC with electrochemical detection at the Jean Mayer USDA Human Nutrition Aging Center at Tufts University, Boston, MA as described previously (22). Briefly, plasma was diluted 10-fold with extraction buffer (0.05 mol/L potassium tetraborate, 1% sodium ascorbate, pH 9.2), heat extracted (100°C for 15 min), and centrifuged for 15 min at 36,000 × g. The supernatant fraction (2 mL) was injected onto the affinity column (10 × 4.6 mm) that contained purified milk folate binding protein covalently bound to AffiPrep 10 support (Bio-Rad). After the affinity column was washed sequentially with 0.05 mol/L potassium phosphate, pH 7, and water, the folates were eluted onto the analytical column (Betasil Phenyl, 250 × 4.6 mm; Keystone Scientific) with an acid mobile phase (0.028 mol/L dipotassium phosphate and 0.06 mol/L phosphoric acid in water). Folates then were eluted from the analytical column using the same aqueous mobile phase at a flow rate of 1 mL/min for 6 min followed by a linear gradient over 50 min to the same mobile phase containing 20% acetonitrile (v:v). This elution separates folates according to both their pteridine ring structure and the number of glutamate residues. Plasma folate forms eluted in the order of 5-methyl-THF followed by FA (pteroylglutamate). Folate activity was determined using an ESA Four Channel Coularray Detector with channels 1–4 set at 0, 300, 500, and 600 mV, respectively. Quantification and identification of individual folates were done by comparison with external folate standards of known concentration.

NK Cytotoxicity. Blood was drawn from fasting subjects between 0730–0830 at the University of Washington under observance of strict criteria [described in reference (23)] that excluded the presence of any infectious symptoms, or the possible influence of other factors known to affect immune function (exercise, sleep patterns, use of nonsteroidal or immunosuppressive medications). All blood draws occurred between May 1998 and July 2000, a time period in which FA fortification had been mandated. Blood samples were processed within 1–2 h of the blood draw; the NK cytotoxicity assay was begun at that time and completed within the same day. A 4-color flow cytometer (XL-MCL, Beckman Coulter) was used to enumerate NK in blood samples, as described previously (23). The flow-cytometric NK cytotoxicity assay used here was also described previously (23), and results correlate well with the chromium release assay. Briefly, mononuclear cells were prepared by Ficoll-Hypaque differential centrifugation of blood effector cells, diluted according to the final effector-to-target (E:T) cell ratios of 50:1, 25:1, 12.5:1, and 6.25:1, and incubated with the DiO-labeled K562 cell suspension (target cells) for 4 h at 37°C with 5% CO_2. After incubation, propidium iodide (PI, 0.03 g/L final concentration) was added to each tube to identify dead cells. The percentage of dead target cells (i.e., dual positive for DiO and PI) among total DiO-identified target cells was used as the measure of NK cytotoxicity. Each assay was performed in duplicate and with appropriate controls. We repeated the NK cytotoxicity assay in 13 study participants who underwent additional blood draws between 1 wk and 9 mo after the initial blood draw, using identical blood draw criteria. Intraclass correlation coefficients between the initial and repeat blood draws were: r = 0.84 (E:T 6.25:1), r = 0.91 (E:T 12.5:1), r = 0.90 (E:T 25:1), r = 0.79 (E:T 50:1), demonstrating high reproducibility. We used the intermediate dilutions (E:T 12.5:1 and 25:1) in our analysis because they showed the highest reproducibility ($r \geq 0.90$) and were in the linear range of the NK cytotoxicity curve; NK cytotoxicity frequently reached a plateau at the 50:1 ratio, and there was little target-cell cytotoxicity at the 6.25:1 ratio.

Statistical analysis. Regression analysis was used to investigate associations between dietary intakes, plasma folate or FA, and NK cytotoxicity, adjusting for potential confounding factors (see below). NK cytotoxicity (E:T 25:1 and 12.5:1) was investigated as a paired measurement; we also fitted models including all 4 E:T ratios and confirmed similar results and identical trends. The paired NK cytotoxicity measurements were analyzed via the Generalized Estimating Equation, accounting for the potential within-person correlation of the 2 NK cytotoxicity measurements (29). Exposures of interest were dietary and supplemental intakes of folate or FA, and subsequent plasma concentrations of unmetabolized FA, 5-methyl-THF, and total plasma folate, which were entered into regression models as categorical variables (tertiles) to allow a nonlinear trend. We also investigated the association between unmetabolized FA, 5-methyl-THF, and total plasma folate both as continuous linear variables and grouped by approximate tertiles. All models were adjusted for factors that were associated previously with NK cytotoxicity in this population: age (continuous), education (3 categories), employment (3 categories), income (3 categories), race (2 categories), energy intake (continuous), and multivitamin use (yes/no). Because immune function declines with age, we also investigated the associations stratified by age group (50–59 and 60–75 y). The percentage (relative) difference was computed by comparing adjusted means of NK cytotoxicity for ET 12.5 and ET 25:1. Analyses were performed using SAS version 8.02 (SAS Institute). Values in the text are means ± SD.

TABLE 1

Characteristics of the study population

Characteristic	
Age, *y*	60.2 ± 6.6
BMI, *kg/m²*	30.3 ± 3.9
Race/Ethnicity, *n* (%)	
Caucasian	93 (89)
Non-Caucasian	11 (11)
Energy intake,[2] *kcal/d*	1678 ± 614
Dietary folate intake, *μg/d*	304 ± 126
Supplemental FA intake, *μg/d*	255 ± 285
Multivitamin use, *n* (%)	57 (54)
Plasma FA, *nmol/L*	2.31 ± 1.91
Plasma 5-methyl-THF, *nmol/L*	42.7 ± 20.8
Plasma total folate, *nmol/L*	45.0 ± 21.1
NK cytotoxicity, 12.5:1 E:T ratio	20.1 ± 12.3
NK cytotoxicity, 25:1 E:T ratio	27.2 ± 13.6
NK, *n*	160 ± 98

[1] Values are means ± SD or n (%), $n = 105$.
[2] To convert to kJ/d, multiply by 4.184.

RESULTS

Characteristics of the study participants are described in Table 1. All women were postmenopausal and overweight or obese (BMI ≥25.0 mg/kg^2), with 49% classified as obese (BMI ≥30.0 mg/kg^2). Per study exclusion criteria, none were taking hormone-replacement therapy or were current smokers. Multivitamins were used by 54% of the study population and 3% used FA supplements. Characteristics of the study population did not differ depending on the presence of unmetabolized FA in their plasma.

Unmetabolized FA was detected in the plasma of 78% of the women in this study. Concentrations of plasma folate metabolites were 2.29 ± 1.91 nmol/L for unmetabolized FA, 42.7 ± 20.9 nmol/L for 5-methyl-THF, and 45.0 ± 21.2 nmol/L for total folate. The presence and concentration of plasma unmetabolized FA were not correlated with plasma total folate.

Our analyses of unmetabolized FA in plasma were prompted by an initial observation of an apparent inverse U-shaped relation between dietary folate or supplemental FA and NK cytotoxicity (**Fig. 1**). For this analysis between source and dose of folate and NK cytotoxicity, we dichotomized dietary folate at <233 μg/d (equivalent to the lowest tertile of folate intake in this population); further, we categorized supplemental FA use into 0 μg/d, 0–400 μg/d, and >400 μg/d, reflecting common amounts used in nutritional supplements. Among women with a low dietary intake (<233 μg folate/d), supplemental folate

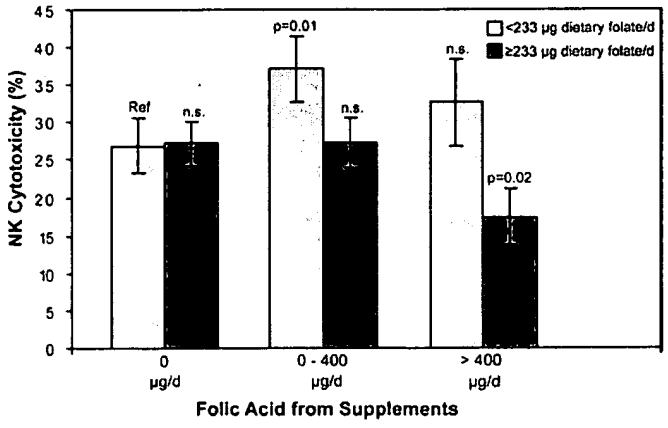

FIGURE 1 Association between FA intake from supplements and NK cytotoxicity among postmenopausal women, stratified by folate intake from diet. NK cytotoxicity is displayed at an E:T cell ratio of 25:1; bars represent adjusted means ± SEM, $n = 105$. In the presence of low dietary folate, modest supplementary FA appears to increase NK cytotoxicity; in the presence of higher dietary folate, high supplementary FA may suppress NK cytotoxicity.

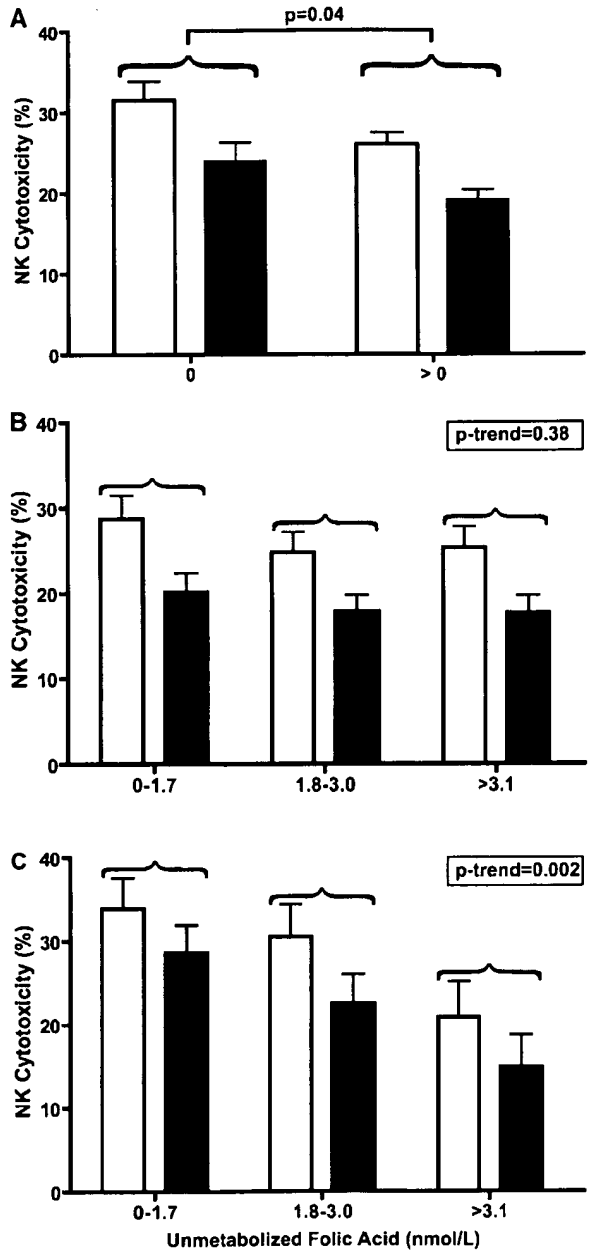

FIGURE 2 NK cytotoxicity among postmenopausal women stratified by the presence of unmetabolized FA in plasma. NK cytotoxicity is displayed at the E:T cell ratios of 25:1 (black bars) and 12.5:1 (white bars). Bars represent adjusted means ± SEM, $n = 105$. *P*-values are derived from a combined analysis of both E:T ratios by multivariable generalized estimating equation. Panels represent: (*A*) all women, any plasma FA vs. none; (*B*) women 50–59 y old, approximate tertiles of plasma FA; (*C*) women 60–75 y old, approximate tertiles of plasma FA. Overall, NK cytotoxicity was lower in women with detectable unmetabolized FA in plasma, an association explained largely by the pattern seen for older women in which there was a significant inverse trend.

intake up to 400 μg/d was associated with higher mean NK cytotoxicity ($P = 0.01$); however, women who had greater dietary intakes (>233 μg/d) did not show additional benefits if they also consumed supplemental FA. Concern arose over our observation that women with higher dietary intakes of folate had significantly lower NK cytotoxicity if they also consumed supplemental FA in excess of 400 μg ($P = 0.02$).

In light of this observation, we subsequently investigated the relation between the presence of unmetabolized FA and NK cytotoxicity. NK cytotoxicity was significantly lower in women for whom unmetabolized FA was detected in plasma (**Fig. 2A**). In the multivariable-adjusted regression models among all study participants, NK cytotoxicity (%) was 6.2% lower in women with unmetabolized plasma FA compared with those without plasma FA present ($P = 0.04$). This corresponds to a relative difference of ~23% (e.g., the ratio of the mean NK cytotoxicity between women with and without detectable FA was 0.77) (Table 2).

When stratified by age (age 50–59 vs. 60–75 y), a strong inverse association between plasma FA and NK cytotoxicity was apparent among women 60–75 y old (Fig. 2C). Mean NK cytotoxicity decreased significantly and linearly with higher concentrations of unmetabolized FA (P-trend = 0.002), resulting in a relative difference of ~25% lower NK cytotoxicity among women with plasma concentrations >3.1 nmol/L compared with those without detectable unmetabolized FA. The associations between unmetabolized FA and NK cytotoxicity remained significant when 5-methyl-THF or plasma folate were simultaneously included in the model, thus demonstrating that the association was independent of these other plasma concentrations (Table 2).

Among women 50–59 y old, the presence of FA was also associated with lower NK cytotoxicity but this was not statistically significant (Fig. 2B). Although NK cytotoxicity was reduced within the intermediate category of 5-methyl or total plasma folate, there were no clear trends for 5-methyl-THF in either age group, suggesting that this was a chance association.

No statistically significant associations were observed in corresponding analyses when the absolute number of NK cells ($CD3^-CD15^+CD56^+CD45^+$) rather than NK cytotoxicity was used as an outcome.

DISCUSSION

In the present study, unmetabolized FA was present in the circulation of 78% of women in the study population. Natural dietary folates occur in reduced and substituted forms of the vitamin, whereas FA, the synthetic form of the vitamin, is fully oxidized and unsubstituted.

Ingested FA can be converted to its physiological forms. This process is initiated by dihydrofolate reductase (DHFR) in a two-step reaction; the first step, conversion to dihydrofolate (DHF), is a slow and rate-limiting step (30). In the second, more rapid, step DHF is further reduced to tetrahydrofolate (THF). THF can then be converted into additional physiological folates including 5-methyl-THF, the form that is normally found in the circulation (31). The human intestine contains DHFR. However, the capacity of this enzyme is limited and when supplemental FA is in excess, a large proportion of ingested FA appears in its unmetabolized form in blood (20,21). Eventually, however, the unmetabolized FA is converted to the reduced forms of folate by peripheral tissues.

In the present study, which used a highly sensitive methodology, FA was detectable in plasma collected from individuals after an overnight fast. In most cases, the amount of FA represented only a small fraction of total folate, which was otherwise comprised exclusively of 5-methyl-THF. In other studies that measured plasma FA, the concentrations were much higher than those seen in the present study (20,21). This difference is likely due to the use of plasma samples from nonfasting subjects after the ingestion of excess FA. Our study population of well-educated postmenopausal women consumed more multivitamins and other supplements than the average

TABLE 2

NK cytotoxicity across tertiles of folate or FA among postmenopausal women stratified by age[1,2]

	All women			Age 50–59 y			Age 60–75 y		
	n	Difference (β)	*P*-value	*n*	Difference (β)	*P*-value	*n*	Difference (β)	*P*-value
FA, *nmol/L*									
0–1.7	34	Ref.	Ref.	17	Ref.	Ref.	17	Ref.	Ref.
1.8–3.0	38	−2.30	0.43	23	−3.10	0.40	15	−9.13	0.07
3.0+	33	−5.12	0.09	20	−2.96	0.36	13	−12.83	0.001
(*P*-trend)			(0.09)			(0.38)			(0.002)
5-Methyl-THF, *nmol/L*									
0–31.9	35	Ref.	Ref.	19	Ref.	Ref.	16	Ref.	Ref.
32.0–51.9	37	−2.90	0.30	25	−6.00	0.04	12	−3.63	0.57
52.0+	33	4.48	0.17	16	2.55	0.48	17	−0.19	0.98
(*P*-trend)			(0.25)			(0.50)			(0.79)
Total plasma folate, *nmol/L*									
0–34.9	31	Ref.	Ref.	16	Ref.	Ref.	15	Ref.	Ref.
35.0–53.9	40	−4.86	0.09	27	−8.09	0.02	13	−5.05	0.43
54.0+	34	2.90	0.39	17	0.63	0.86	17	−0.52	0.93
(*P*-trend)			(0.47)			(0.64)			(0.66)

[1] Combined analysis of E:T ratios 25:1 and 12.5:1 by generalized estimating equation, *P*-values in comparison to referent (Ref.) group.

[2] Models adjusted for age (continuous), education (3 categories), employment (3 categories), race (2 categories), energy intake (continuous), and multivitamin use (yes/no).

U.S. population (13), which may help explain the high prevalence of circulating plasma FA in this population.

Our data showing that unmetabolized FA in plasma is associated with decreased NK cytotoxicity are a cause for concern. This association with FA was independent of circulating 5-methyl-THF and total folate. The association was strong and significant among women 60–75 y old. This is consistent with findings showing that immune function may be more easily modulated among the elderly (32).

Nutritional factors such as vitamin E, zinc, and multivitamin supplements are widely recognized as important determinants of immune function (32). However, few studies have examined the relation of folate to immune function in general and to NK cytotoxicity in particular. Kim et al. (33) showed that folate deficiency can diminish NK cytotoxicity in rats, findings that are consistent with our findings of greater NK cytotoxicity with FA supplement use among women with low dietary folate intakes. A study in an Italian population of healthy 90- to 106-y olds found no correlation between plasma *total folate* concentration and NK cytotoxicity (34). However, as mentioned above, the decrease that we observed in NK cytotoxicity in association with unmetabolized plasma FA was independent of total folate and 5-methyl-THF concentrations. Subjects in the Italian study did not use nutritional supplements, and thus plasma *folic acid* was not measured.

NK cells are part of the nonspecific immune response and can kill a variety of normal and virus-infected cells without prior sensitization. Experimental and clinical evidence supports a role of NK cells in tumor cell destruction; thus, this component of the immune system may be considered a first line of host defense against carcinogenesis (19). Although the relation between in vitro NK cytotoxicity and in vivo cytotoxicity is incompletely understood, decreased NK cytotoxicity may increase the risk or severity of infections and was associated with increased future cancer incidence in a Japanese cohort study (35). If excess folate does in fact suppress NK cytotoxicity in vivo, then this would suggest another way in which excess folate might promote existing premalignant and malignant lesions.

At present, we lack a clear mechanistic explanation for our observation. Preliminary data from mathematical modeling indicate that very high folate intakes may create biochemical conditions similar to those of folate deficiency (36). Our study raises concern about possible direct toxicity of FA, but it does not exclude the possibility of an underlying biologic defect among some women, which may result in the appearance of unmetabolized FA in plasma and concurrently have adverse effects on NK cytotoxicity. Although our study was prompted by initial observations of lower NK cytotoxicity among women with high folate supplementation, we did not observe a correlation between dietary/supplementary FA intake based on the questionnaire data and measured plasma FA concentrations. It is possible that a low capacity to metabolize large amounts of FA might be a metabolic correlate of immune function. To explore this possibility, the metabolic and functional effects of polymorphisms in the *DHFR* gene (37,38), and their relation to immune function merit further investigation.

Much of our knowledge of the relation between folate and the immune system derives from studies of nutritional deficiencies or the use of antifolate drugs. Few studies were conducted on the condition of high folate intake. The possibility of an adverse effect of excess total folate or FA intake in humans, in the range of current population levels of dietary intake, was raised recently in relation to a possible adverse effect on cognitive function (39). Further, several studies in rats showed adverse effects of excess FA. Although dietary FA enrichment of 8 mg FA/kg diet (3 times the recommended levels for rodents) did not diminish NK cytotoxicity in rats, diets containing 40 mg FA/kg diet (20 times the recommended dietary folate concentration for rats) accelerated cancer progression in rodent models of cancer (18,40,41). More recent studies found that dietary intake of 40 mg FA/kg diet reduced birth weight and size in the offspring of pregnant rats consuming high FA compared with rats consuming a control diet (2 mg/kg). High dietary FA was also associated with elevations of the methyl donor *S*-adenosyl-methionine and decreases in the efficiency of nitrogen metabolism (42–44). Finding low NK cytotoxicity in elderly people with unmetabolized FA in the circulation is a concern that has possible public health implications.

This study highlights the need for a better understanding of the relation of folate metabolism, immune function, and health. Considering the increased intake of FA in the population (10,11), our finding of an adverse relation between circulating FA and NK cytotoxicity must be corroborated in larger studies. Until additional studies are conducted, calls for further increases in food FA fortification and intake should be viewed with caution.

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